Succession of Esterase and Peroxidase Isozymes Associated with the in vitro *Sugarcane Tissue Dedifferentiation and Shoot Induction*

✍ Guillermo Carrillo-Castañeda, Anastacia Mata

Colegio de Postgraduados. IREGEP. Montecillo, Méx. 56230. México.
E-mail: carrillo@colpos.colpos.mx

ABSTRACT

Isozyme patterns expressed by subcultured calli can be used as markers to determine arbitrary identifiable stages along the process of cellular dedifferentiation which affects callus totipotency. Calli initiated from leaf tissue of sugarcane *(Saccharum* spp.) plants (varieties *ZMéx.55-32* and *Méx.57-473)* were subcultured at 30-day intervals (stages) on MS dedifferentiation medium containing 3 mg/L 2,4-dichlorophenoxiacetic acid (2,4-D) for up to 10 stages. Calli from each of the 10 stages were transferred to 2,4-D free MS medium, where they were subcultured for two periods of 30 days each to assess their morphogenetic ability. The dedifferentiated calli subcultured for up to 6 stages were able to express their full morphogenetic competence which gradually decreased up to 8% at the end of the 10th stage. In terms of molecular markers, donor plant leaf tissue of both varieties expressed 2 esterases and 3 peroxidases; however, in the first 3 stages along the cell dedifferentiation process, in which the transition of leaf tissue to callus takes place, up to 6 esterases and 12 peroxidases were detected from a total of 17 esterases and 23 different peroxidases expressed by the calli along 9 subcultures. Peroxidase patterns showed the highest differential expression, which allowed us to distinguish between the 9 dedifferentiation stages under study. Cluster analysis of esterases allowed us to differentiate the expression in the first 3 stages from the subsequent ones. Peroxidases showed a more complex phenogram.

Keywords: cell dedifferentiation, isozymes, *Saccharum,* shoot induction

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RESUMEN

Sucesión de esterasas y peroxidasas asociadas a la desdiferenciación celular e inducción *in vitro* de brotes de caña de azúcar. Los patrones de isoenzimas expresados por callos subcultivados, pudieran ser usados como marcadores para determinar estadios de desdiferenciación arbitrarios identificables a lo largo del proceso de desdiferenciación celular que tiene relación y afecta la totipotencia de los callos. Callos establecidos en el medio de desdiferenciación MS con 3 mg/L de ácido 2,4-dichlorophenoxiacético (2,4-D) a partir de tejido foliar de plantas de caña de azúcar *(Saccharum* spp.), variedades *ZMéx.55-32* y *Méx.57-473,* fueron subcultivados por periodos consecutivos de 30 días cada uno (estadios) durante 10 estadios. Callos de cada uno de los estadios fueron transferidos al medio MS sin 2,4-D, donde fueron subcultivados durante dos periodos de 30 días cada uno para evaluar su capacidad de formación de brotes. Hasta el sexto estadio, los callos expresaron la máxima capacidad organogénica, la cual disminuyó hasta 8% al final del décimo estadio. En relación con los marcadores moleculares, el tejido foliar de las plantas donadoras de ambas variedades expresó 2 esterasas y 3 peroxidasas, pero en el proceso de desdiferenciación, durante la transición de tejido foliar a callo, que ocurre en los primeros 3 estadios, se detectaron hasta 6 esterasas diferentes y 12 peroxidasas, de un total de 17 esterasas y 23 peroxidasas diferentes expresadas a lo largo de 9 subcultivos consecutivos. Los patrones de peroxidasas mostraron la máxima expresión diferencial lo que permitió distinguir entre los 9 estadios de desdiferenciación. El análisis de agrupamiento por afinidad permitió diferenciar las expresiones de esterasas de los 3 primeros estadios de las subsecuentes, pero el agrupamiento de las peroxidasas mostró un patrón más complejo.

Palabras claves: desdiferenciación celular, inducción de brotes, isoenzimas, *Saccharum*

*I*ntroduction

More than thirty years ago, Heinz and Mee described protocols to establish sugarcane callus cultures, as well as a high frequency plant regeneration [1]. This process involves the transformation of somatic cells into a plant via cellular dedifferentiation and redifferentiation. Authors have attempted to elucidate the underlying biochemical and molecular mechanisms of cell differentiation. In spite of the fact that it has been demonstrated that the undifferentiated condition of calli (age) is a factor that affects cell totipotency [2–5], there is rather limited information available regarding the biochemical and molecular changes that accompany the process of cellular dedifferentiation [2, 6, 7] and there is no precise explanation for this callus behavior. Analysis of cellular constituents, such as secondary metabolites, proteins and isozymes from calli developed under specific conditions, provides a useful tool to assess their physiological condition. The oxidase activity of plant peroxidases is modulated by certain phenolics [8] and, as quoted by Ponce *et al.* [9], secondary metabolites gradually decreased in long-term subcultured sugarcane calli. While studying the changes in protein expression during leaf transformation into callus, Ramagopal [10] identified 63 dedifferentiation proteins, which might be useful to characterize biochemical changes that occur during the

✍ Corresponding author

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dedifferentiation process of sugarcane cells. In this context peroxidase isozymes are quite important because they catalyze several physiological reactions, such as indole-3-acetic acid oxidation, a major degradation reaction involved in controlling the levels of this powerful plant hormone *in vivo*, implicated in the regulation of cell growth and development [8, 11] and the adaptation processes of higher plants to environmental stress [12]. Several authors have found a decrease, as well as other changes, in plant regeneration patterns in long-term subcultured calli [2, 6, 13, 14]. This paper is aimed at providing information to bridge the expressions of both esterase and peroxidase enzyme activities that accompany the *in vitro* sugarcane leaf cell dedifferentiation process and the plant regeneration potential in these long-term subcultured calli.

Materials and Methods

Callus establishment, subculture and shoot regeneration

Eight- to 10-month-old field-grown sugarcane plants from cultivars *ZMéx 55-32* and *Méx 57-473* were used in this study. Explants were surface-sterilized by sequential immersion in 70% ethanol for 1 min and sodium hypochlorite (2% free Cl), 0.1% liquid detergent for 30 min, and washed 4 times with sterile distilled water. The *in vitro* callus cultures were established using leaf roll sections of about 3–4 mm thick from the apical region of the stem above the shoot apical meristem, and internode stem tissue from the apical region of plants. The explants were placed on MS3 solid medium (a modification of Murashige and Skoog medium [15], containing 100 mg/L inositol; 1 mg/L thiamine-HCl; 3 mg/L 2,4-dichlorophenoxiacetic acid (2,4-D); 30 g/L sucrose; 180 mL/L coconut milk, and the pH was adjusted to 5.8 before adding 7.5 g agar). All culture media were autoclaved at 121 ºC for 15 min. The cultures incubated at 26–29 ºC and 16 h of light daily were maintained on MS3 medium [16, 17]. All cultures were subcultured every 30 days. Shoot induction was attained after callus cultures were transferred from the MS3 medium onto MS0 (the MS3 medium without 2,4-D), and subcultured for two periods of 30 days each under the conditions here described.

Isozyme analysis

The isozyme analysis was carried out using: 1) internode tissue from the apical region of the stem; 2) the same kind of leaf tissue used to establish the plant tissue cultures; and 3) callus cultures developed on the MS3 medium and harvested at the end of each subculture. In order to prepare the crude extracts, 1 g of the apical region of the stem or leaf tissue from which the *in vitro* cultures were established and the callus cultures developed on MS3 medium, was ground at 4 ºC together with 1 mL of phosphate buffer 0.1 M pH 8 and 0.1 g of ground glass. The debris was spun down at 21 800 x*g* at 4 ºC during 15 min. The clear supernatants were dialyzed at low temperature against phosphate buffer diluted 1:10 with two changes of 2 h each. The extracts were immediately used in the enzymatic assays. The amount of protein in the extracts was determined by the method described by Lowry *et al.* [18].

Series of isozyme determinations were performed monthly to determine their isozyme patterns at every callus stage. A versatile procedure for the routine isozyme analysis was carried out [19] and according to it proteins were separated by horizontal 242 x 120 mm electrophoresis in 10% polyacrylamide gels. Several independent extracts from plant tissue and calli of each variety were prepared. Four samples from each extract were loaded into the wells of the 1.5-mm-thick gel, and electrophoresed (100 V, 50 mA) to determine peroxidase isozymes (E.C. 1.11.1.7) according to Hart *et al*. [20] and esterase isozymes (E.C. 3.1.1) as quoted by Scandalios [21]. The extracts contained equal amounts of protein.

Cluster analysis

The bands were recorded as present (1) or absent (0), and compiled into a two-way matrix (dedifferentiation stages x markers). Dendrograms based on the similarity matrix data were constructed using the Jaccard's index and applying the unweighted pair group method with arithmetic averages (UPGMA) cluster analysis. The Numerical Taxonomy and Multivariate Analysis System program package for PC (NTSYS-PC version 1.8) [22] was used.

Results

Callus and shoot induction

Leaf and stem tissues from both varieties at the end of the third subculture on MS3 medium suffered a drastic transformation into calli. The results of four independent experiments using 60 explants per experimental condition per experiment showed that callus cultures were established in 88% and 97% of the *ZMéx.55-32* and *Méx.57-473* leaf explants, respectively. This ability was slightly lower in stem explants: 80% in *ZMéx.55-32* and 75% in *Méx.57-473*. Based on these results, leaf tissue explants were used to continue this research.

Shoot induction was first assayed in 4-month-old callus tissue; at least 40 per variety were transferred from MS3 to MS0 medium and subcultured for two periods of 30 days each. Thereafter shoots were detected in 50–54% of the callus cultures from the two varieties. Figure 1A shows the aspect of normal plantlets obtained from these calli under these experimental conditions. Field trials of these kind of plants, conducted to assess their performance based on 15 phenotypic traits, indicated that their behavior was quite normal [23].

Callus cultures initiated from leaf tissue on MS3 medium were subcultured at 30-day intervals for up to 10 stages. After calli from each stage were transferred to MS0 medium and subcultured twice, the morphogenetic competence was scored (Figure 1B). Under these experimental conditions these callus cultures expressed their full morphogenetic capacity for up to 6 stages, and from the 7th stage they exhibited a gradual decrease which dropped to 8% at the end of the 10th month.

Isozymes patterns changes during dedifferentiation

Peroxidase and esterase isozyme expressions of the original plant tissue and callus cultures, established

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from leaf tissue and subcultured on MS3 medium at 30-day intervals (stages), were determined at the end of each of the nine consecutive stages.

Esterases. Two esterase activities that differed in their electrophoretic mobility were present in both the leaf and the stalk tissues. These patterns appeared to be quite similar in both varieties (Figure 2A). The *in vitro* cultures along the 9 stages, however, expressed a total of 17 *(ZMéx.55-32)* and 16 *(Méx.57-473)* different species (Figures 2B and 2C). Many of them

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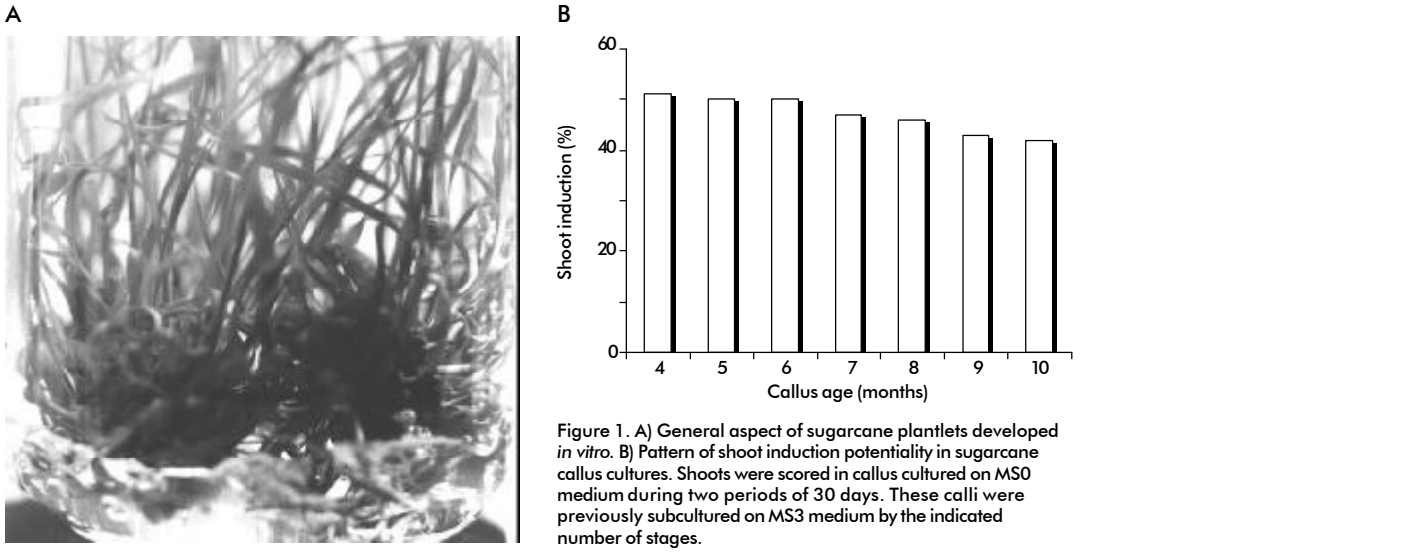

Figure 1. A) General aspect of sugarcane plantlets developed *in vitro*. B) Pattern of shoot induction potentiality in sugarcane callus cultures. Shoots were scored in callus cultured on MS0 medium during two periods of 30 days. These calli were previously subcultured on MS3 medium by the indicated number of stages.

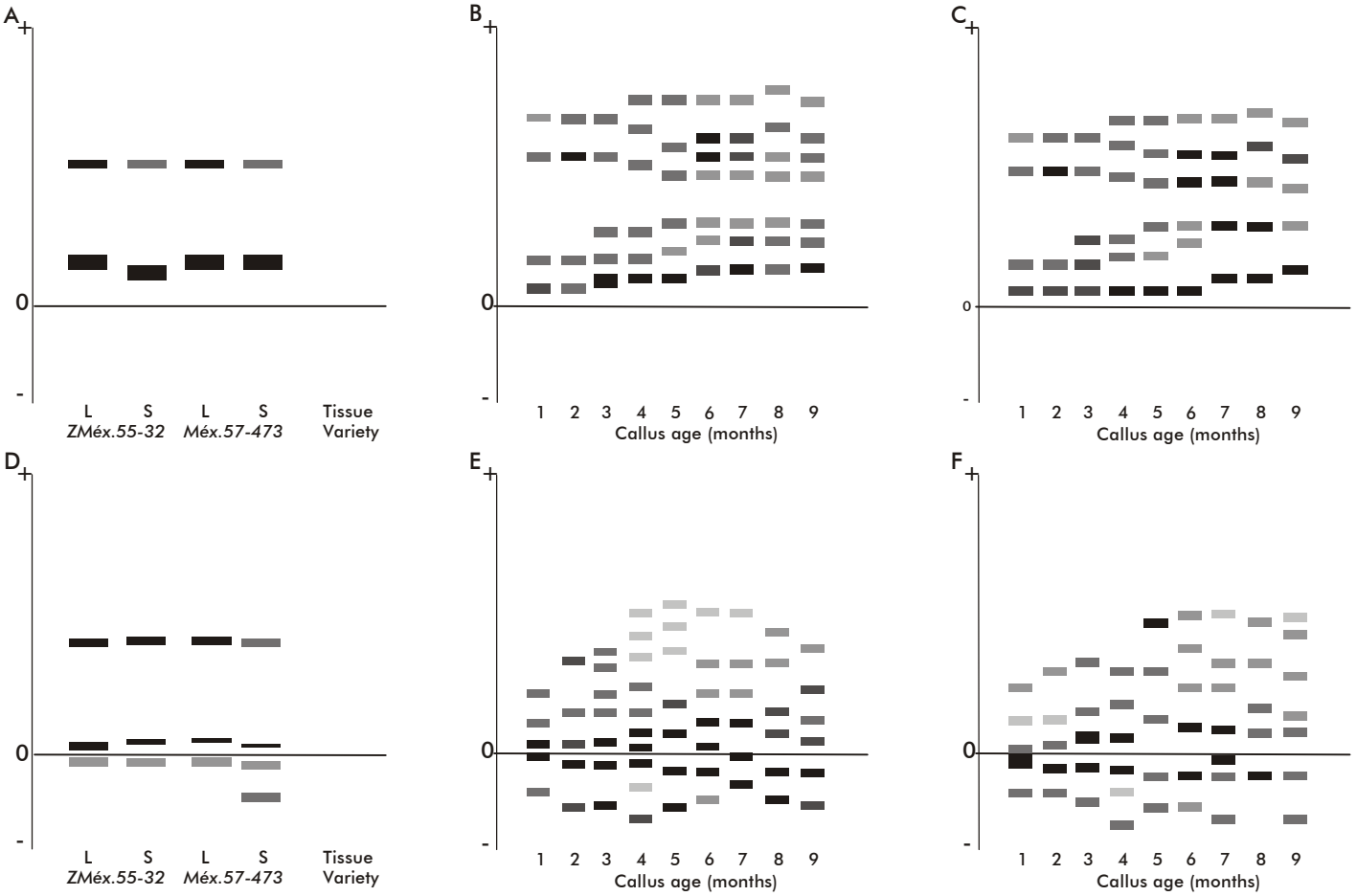

Figure 2. Isozyme zymograms from *Saccharum* spp. plant tissue (L, leaf; S, stem) and calli subcultured at one-month intervals on MS3 culture medium: plant tissue esterases (A) and peroxidases (D); calli esterases of *ZMéx.55-32* (B) and *Méx.57-473* (C); and calli peroxidases of *ZMéx.55-32* (E) and *Méx.57-473* (F). Differences in broadness and color intensity of bands was apparent: intense (black), light (white).

appeared in more than one stage (Figures 3A and 3B) and only 6 species differed in their relative electrophoretic mobility between these varieties. The highest number of species detected by stage in *ZMéx.55-32* was 7, which were expressed from the sixth to the ninth stage; 6 were expressed in *Méx.57-473* from the fourth to the sixth stage. Variations in the intensity of the protein bands on the zymograms were observed among the extracts from different calli. All expressed esterases migrated to the cathode.

The analysis of the UPGMA clustering method showed a separation of the 9 stages into 2 main branches of the dendrograms (Figures 4A and 4B). The enzyme expressions of the first 3 stages—period in which occurs the transformation of leaf tissue into callus—were clustered together in the first subgroup. In the second branch we found major differences between the two sugarcane varieties.

Peroxidases. A higher number of peroxidase activities occurred during cell dedifferentiation in both varieties in comparison with the esterase isozyme patterns. Leaf tissue from both varieties expressed 3 species of peroxidases, while in stalk tissue, 3 and 4 species appeared in *ZMéx.55-32* and *Méx.57-473*, respectively (Figure 2D). In the first 3 stages, period in which leaf tissue was transformed into callus, 11 and 12 different peroxidases were expressed in the calli from *ZMéx.55-32* and *Méx.57-473*, respectively, of the total of 23 different activities that were expressed in both varieties. Few peroxidases migrated to the anode (Figures 2E and 2F) and many of them were present in more than one stage (Figures 3C and 3D). From the fourth subculture on, a higher number of species and a greater difference in the isozyme patterns between these two sugarcane varieties were observed.

The dendrograms displayed by the tree program obtained for these enzyme expressions (Figures 4C and 4D) indicated that the expression of these enzymes during the first 3 stages had, in comparison with the expressions of esterases, a higher degree of dissimilarity, since they did not cluster together.

Discussion

Callus cultures

As far as plant cell dedifferentiation was concerned, the explants from both varieties under study exposed to the presence of exogenous 2,4-D showed a similar callus induction potentiality. Nevertheless, it is well known that certain capabilities, enzymatic activities and proteins that are specifically expressed by plant tissues, are gradually lost in long-term cultured calli, and these expressions might be related to gene regulation determined by the bio-

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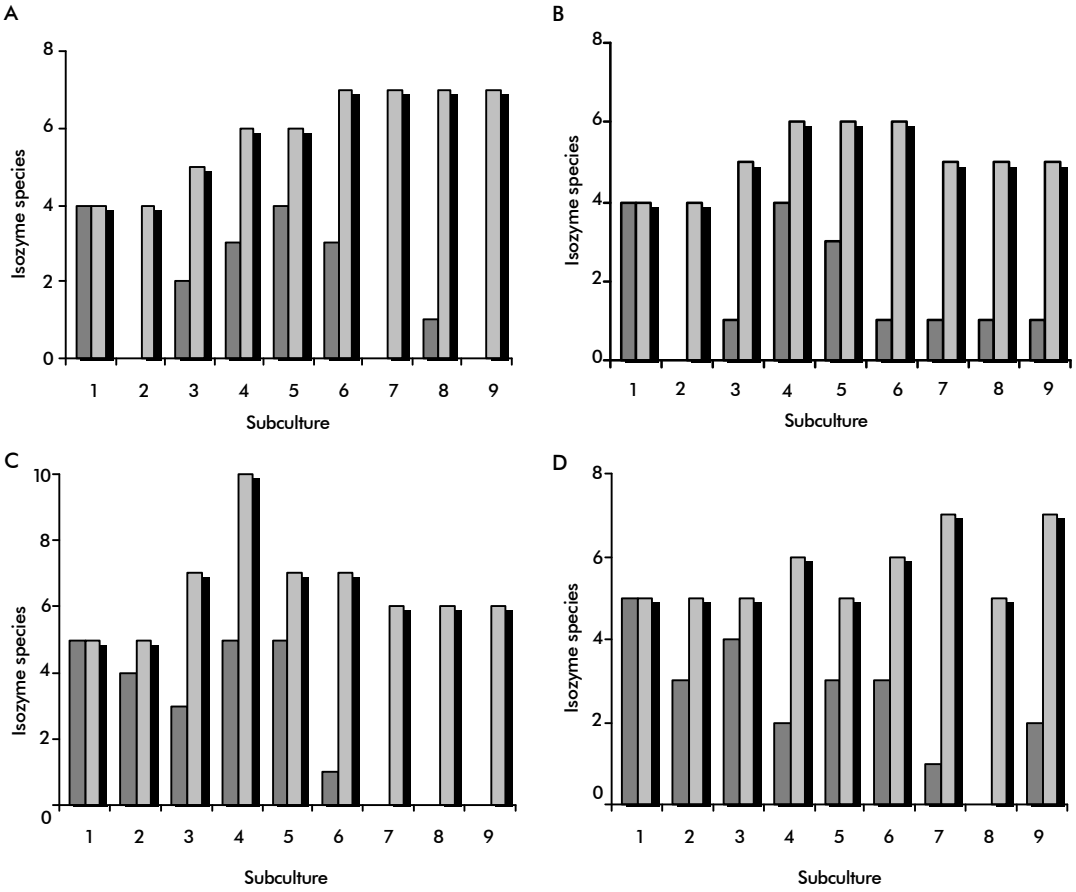

Figure 3. Total number (light bars) and newly expressed isozyme species (solid) in each stage in *Saccharum* spp. calli subcultured at one-month intervals on MS3 medium. Esterases from: *ZMéx.55-32* (A) and *Méx.57-473* (B); peroxidases from: *ZMéx.55-32* (C) and *Méx 57-473* (D).

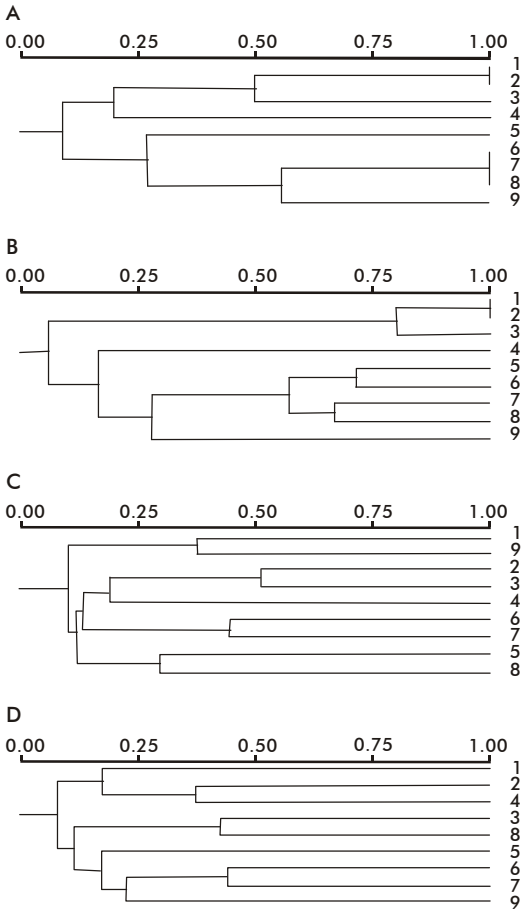

Figure 4. Relationships among nine sugarcane cell dedifferentiation stages determined by their enzymatic similarities estimated by cluster analysis using UPGMA with Jaccard's coefficient (NTESYS-PC version 1.8). Esterase isozymes dendrograms from *ZMéx.55-32* (A) and *Méx.57-473* (B); peroxidase isozymes dendrograms from *ZMéx.55-32* (C) and *Méx.57-473* (D).

chemical conditions of the cells along the dedifferentiation process and of the plant genotype [2, 3, 9, 17, 19, 24].

We demonstrated that an early event observed in the cells facing the transition from plant tissue to callus was a burst of both isozyme expressions in the two sugarcane varieties, which may be the consequence of several facts: 1) the *in vitro* cell dedifferentiation process overcomes the natural tissue-specific control over sets of enzyme expression in these cells; 2) specific gene expression adjustments may be involved, allowing calli to become fully adapted to artificial culture conditions; and 3) cells under stress may concomitantly induce altered enzymatic activities [12, 25].

In the case of esterases, cluster analysis based on the enzyme expression similarities of both varieties placed the first 3 stages into a separate cluster, which suggests that the pattern of enzyme activities performed in the cells during the transition of the leaf tissue to callus are different from the enzyme expression detected in subsequent stages. Our results indicate that peroxidase activities showed a more complex pattern, since, in principle, a higher number of different species were expressed, and due to a lower similarity of the expression patterns among the first 3 stages in which they did not cluster together. In fact, plants may usually express between 8 and 15 peroxidase families, an ubiquitous enzyme whose expression is scattered in different organs, as well as in the chloroplast and cytosol [26]. Thom and Maretzki [27] analyzed the variations of peroxidase and esterase isozymes in different tissues of several sugarcane varieties, and demonstrated that the peroxidase isozyme patterns in a given plant tissue are specific for each clone, and that the differences in isozyme patterns from different tissues reflect the metabolic specificity within the plant tissues. In addition, this activity is indirectly involved in the regulation of plant cell growth, and abnormal peroxidase activity induced in the cells under stress may be accompanied by changes in their development [25]. Thus, the spectrum of peroxidase isozyme patterns found along the first 3 stages (transition from leaf tissue to callus) showed that 17 and 15 peroxidase isozymes were expressed in *ZMéx.55-32* and *Méx.57-473*, respectively, which is about 50% of the total different species found along the 9 stages under analysis. The study of the changes in protein expression along the transformation of leaf tissue into callus—which presumably could be used to characterize the biochemical, molecular, and genetic properties of sugarcane cultures—evidenced that, depending on the species, the succession of dedifferentiation proteins is regulated by both qualitative and quantitative mechanisms that are apparently programmed by certain genes [10]. Pointing in the same direction, we have demonstrated that these isozyme patterns are reproducible. In an independent experiment the enzymatic expressions in 1- and 7-month-old callus cultures of these varieties, showed that the isozyme patterns in all these cases were essentially the same, as compared with the corresponding ones previously obtained.

Shoot induction

Totipotency, a key phenomenon that relies upon cell division and cell dedifferentiation, has allowed to establish a feasible link between fundamental biology and the practical use of biotechnology. Shoot induction was observed in about 50% of the callus cultures in the absence of exogenous regulatory substances, which suggests that a precise balance of the endogenous regulatory substance is required for the redifferentiation induction and plant micropropagation. Fitch and Moore [6] showed that the total number of regenerated shoots decreased with time in the long-term culture of embryogenic sugarcane calli, while the number of pale green plants increased after five months of culture. Our callus cultures capable of regenerating normal plants after the long-term subculture process showed a gradual decrease in their cell redifferentiation potentiality from the 7th subculture on. Abe and Futsuhara [13] stated that selected rice calli that redifferentiated numerous shoots showed a more intense peroxidase band, compared with calli that showed a poor regeneration potentiality. They suggested that the ability of rice callus to form shoots was related to the peroxidase isozyme pattern. The secretory plant per-

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oxidases catalyse tissue-specific reactions involved in indole-3-acetic acid catabolism, cell wall biosynthesis, ethylene biosynthesis, oxidation of toxic compounds, and removal of hydrogen peroxide from chloroplasts and cytosol [28, 29]. Changes in enzymatic activities that modify the delicate equilibrium among plant cell regulators in callus cultures may alter their redifferentiation patterns [30]. Indole-3-acetic acid is indeed an important plant growth regulator, and its oxidative decarboxylation by guaiacol-type plant peroxidases is thought to control the *in vitro* level of this plant hormone [31].

The lack of ability to detect genetic changes using RAPD assessment of plants regenerated from *in vitro* long-term cultures revealed a genetic stability [32, 33]. In the same direction, the analysis of sugarcane plants regenerated from callus tissue, based on 15 phenotypic traits, revealed no significant phenotipic changes [23]. *In vitro* culture techniques have been developed for the conservation of *Saccharum* spp. hybrid germoplasm [34].

We are focusing our attention on the identification—based on isozyme markers—of the experimental conditions required to enhance the long-term retention of a high frequency of shoot induction in sugarcane calli for the competitive clonal plant production.

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